PARALYSIS IN BOTULINUM TOXIN POISONING

An experimental study on pathophysiology and drug treatment

By

Håkan Lundh

Lund 1978

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This thesis is based on the following publications, which will be referred to by their Roman numerals in the text.

I Cull-Candy SG, Lundh H, Thesleff S: Effects of botulinum toxin on neuromuscular transmission in the rat. J Physiol (Lond) 260:177-203, 1976

II Lundh H, Schiller HH, Elmqvist D: Correlation between single fibre EMG jitter and endplate potentials studied in mild experimental botulinum poisoning. Acta Neurol Scand 56:141-152, 1977

III Lundh H, Leander S, Thesleff S: Antagonism of the paralysis produced by botulinum toxin in the rat. J Neurol Sci 32:29-43, 1977

IV Lundh H: Effects of 4-aminopyridine on neuromuscular transmission. Brain Research 1978 in press

V Lundh H, Nilsson O, Rosén I: 4-Aminopyridine - a new drug tested in the treatment of Eaton-Lambert syndrome. J Neurol Neurosurg Psychiat 40:1109-1112, 1977

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INTRODUCTION

1. Disorders of neuromuscular transmission

Disturbances of neuromuscular transmission causing muscle paralysis may result from interruption of any one of the physiological steps underlying synaptic transmission. Neuromuscular block may occur if transmitter release from nerve terminals is diminished, as in botulinum toxin poisoning (Burgen, Dickens & Zatman 1949) and the Eaton-Lambert syndrome (Elmqvist & Lambert 1968), or if the acetylcholine sensitivity of the muscle membrane is decreased, as is the case in curare poisoning and probably in myasthenia gravis (Fambrough, Drachman & Satyamurti 1973, also see Grob 1976). Neuromuscular block may also occur as a result of abnormalities in endplate acetylcholinesterase activity as in poisoning by insecticides and warfare agents and in a recently recognized human disease (Engel 1977). In all these conditions, the endplate potentials (epps) produced by the acetylcholine action on the postsynaptic membrane are subthreshold and unable to trigger muscle action potentials and muscle contraction.

In recent years the postsynaptic disturbance of neuromuscular transmission demonstrated in myasthenia gravis has been extensively investigated, and a specific defect of junctional physiology has been demonstrated. Knowledge of the mechanisms involved in presynaptic failure of neuromuscular transmission, on the other hand, has not advanced to the same level of discrimination. The present investigation was performed in an attempt to explore the pathophysiology of diminished transmitter release, and to this purpose, experimental botulinum toxin poisoning was used as a

model of a presynaptic defect. This study elucidated a defect of the transmitter release process at the point where calcium is involved, and the results were used to try to find drugs to antagonize the blockade of transmitter release. It was shown, that drugs which are believed to increase the intracellular concentration of calcium in nerve terminals restored neuromuscular transmission, and experiments on the mode of action of these drugs led to the description of a new type of pharmacological action on nerve terminals. Finally, one of the drugs was tested in man.

2. Botulinum toxin poisoning

Botulinum toxin. Botulinum toxin (BoTx) is liberated into the growth medium upon autolysis of the bacillus Clostridium botulinum. Eight serospecific types of Cl. botulinum toxin have been recognized and animal species differ in susceptibility to the different types. Type A, which is the most poisonous type, was the first one to be obtained in crystallized form (Abrams et al. 1946, Lamanna et al. 1946) and was shown to be a protein with a molecular weight of about 900 000. More recent results (DasGupta et al. 1966) have demonstrated that BoTx is made up of two components, one of which has a molecular weight of about 500 000 and contains the hemagglutinin activity of BoTx (Lamanna 1948), and a second component with a molecular weight of around 150 000 which contains the neurotoxin.

BoTx is considered the most potent of all poisons. One gram contains 20 000 million mouse lethal doses. The lethal dose in man is unknown but may not differ much from that in mice, as it may depend on the number of endplates and not on body weight (Lamanna 1959).

Botulinum toxin poisoning in man. The ingestion of preformed toxin in contaminated food causes human and animal botulism (van Ermengem 1897). Botulism as a disease entity was pointed out by German physicians in

the beginning of the nineteenth century, but it has been claimed (Meyer 1928), that the disease was known already during antiquity. During the first two decades of this century and especially during and after World War I, botulism emerged as a significant public health problem in Europe and the United States. This coincided with the development of the canning industry and an increase of home-canning and preserving of food during the war. Investigations on the properties of Cl. botulinum by Meyer and coworkers (see review by Koenig 1971), especially stressing the destruction of the toxin by heating, soon came to practical usefulness, and the number of cases of botulism has constantly decreased since the 1930s.

The clinical symptoms of human botulism usually start with disturbances of the autonomic nervous system, i.e. a pronounced dryness of the mouth, intestinal constipation and urinary retention. The neurological symptoms are blurring of vision and diplopia, followed by dysphagia and dysphonia, facial weakness, ptosis and mydriasis with loss of the reflex to light. The paralysis may progress and involve the muscles of the neck and the respiratory muscles including the diaphragm causing difficulty in breathing. The muscles of the arms and legs are usually only mildly affected, producing proximal muscle weakness with preserved tendon reflexes, but in some cases marked paralysis of arms and legs have been described. Obviously, all degrees of severity of clinical symptoms occur, and it has been demonstrated that in some cases symptoms are so mild, that the person never comes to medical consultation. Although some reports speak of mental sedation and somnolence in human botulism, this is not usually present, and the patient has preserved mental clarity even in advanced cases of muscle paralysis. The cause of death in fatal cases is usually respiratory failure.

Electrophysiological examination of BoTx poisoned muscles discloses reduced amplitude of the compound muscle action potential, increment of this potential

at high nerve stimulation frequencies, post-tetanic facilitation and fibrillation potentials (Petersén & Broman 1961, Tyler 1963, Cherington & Ginsberg 1971, Gutman & Pratt 1976). These neurophysiological findings are partly similar to those found in the myasthenic syndrome sometimes associated with bronchogenic carcinoma (Eaton-Lambert). Treatment of botulism includes early tracheotomy and mechanical assistance of respiration. The administration of botulinum antitoxin is recommended in all cases, but is probably efficient only in eliminating toxin not yet fixed to tissue binding sites (Guyton & McDonald 1947). In non-fatal cases recovery takes several weeks or months. In recent years the drug guanidine hydrochloride has been demonstrated to be of benefit in reducing the muscle paralysis in human botulism (Cherington & Ryan 1968, 1970). A possible new therapeutic agent pointed out in this investigation is 4-aminopyridine. This drug has been known to pharmacologists for its sympathomimetic actions (von Haxthausen 1955, Fastier & McDowell 1958), and has recently been shown to antagonize the neuromuscular block caused by curare (Lemeignan & Lechat 1967, Molgo, Lemeignan & Lechat 1975).

Animal experiments. BoTx acts by paralysing the parasympathetic nervous system and neuromuscular transmission, i.e. it blocks cholinergic synapses (Dickson & Shevky 1923 a, b, Ambache 1949). The paralysed muscle responds normally to direct stimulation, and impulse conduction in nerve axon and nerve terminals is not impaired (Bishop & Bronfenbrenner 1936, Harris & Miledi 1971). The motor endplate reacts normally to acetylcholine (Thesleff 1960), and the intra-arterial administration of acetylcholine causes normal muscle contraction (Guyton & McDonald 1947). The nerve terminal output of transmitter is diminished, but BoTx has no effect on the synthesis of acetylcholine or on choline esterases (Burgen, Dickens & Zatman 1949).

During onset of paralysis in BoTx poisoning, motor endplate potentials (epps) rapidly decrease in ampli-

tude and finally disappear, and spontaneous miniature endplate potentials (mepps) disappear (Brooks 1956, Thesleff 1960) except for some mepps of reduced amplitude and frequency, which persist even when neurally evoked epps are abolished (Harris & Miledi 1971, Spitzer 1972, Boroff, del Castillo, Evoy & Steinhardt 1974, Tonge 1974). Transmitter release can be temporarily increased by tetanic nerve stimulation (Brooks 1954 & 1956), mechanical irritation of the nerve terminal, increasing the calcium concentration in the bathing fluid or the addition of tetraethylammonium (Thesleff 1960).

When in contact with the muscle, the toxin is rapidly and irreversibly bound to the nerve endings (Burgen, Dickens & Zatman 1949). The onset of paralysis is delayed and dependent on metabolic energy (Burgen et al. 1949) and accelerated by factors which increase transmitter release, i.e. nerve stimulation (Hughes & Whaler 1962, Boroff et al. 1974) and increased calcium concentration (Simpson 1973).

The muscle paralysis in BoTx poisoning is long-lasting and recovery takes several weeks or months (Guyton & McDonald 1947), and depends on the outgrowth of nerve sprouts from the terminal and preterminal parts of motor nerve fibres creating new neuromuscular junctions (Duchen & Strich 1968). The morphological structure of nerve and motor endplate is not altered by BoTx as revealed by electron microscopy (Katz, quoted by Thesleff 1960), but poisoned muscles undergo atrophy similar to that after denervation (Jirmanová, Sobotková, Thesleff & Zelená 1964). Also, BoTx poisoned muscles undergo functional alterations characteristic of denervation, i.e. spread of the acetylcholine sensitive area to the whole muscle membrane (Thesleff 1960), and fibrillation potentials appear (Josefsson & Thesleff 1960).

METHODOLOGICAL CONSIDERATIONS

The experiments summarized in this thesis were performed on the extensor digitorum longus (EDL) muscle of male rats and the sartorius muscle of frogs. One patient suffering from the myasthenic syndrome associated with bronchogenic carcinoma took part in clinical drug tests. This patient had the characteristic clinical and electrophysiological signs of the Eaton-Lambert syndrome.

Rat EDL muscles were poisoned by a single subcutaneous injection of Clostridium botulinum toxin type A. The amount of toxin given produced complete paralysis of the EDL muscle of the injected leg within 18 hours, and the paralysis remained for the whole observation period of three weeks. Doubling the dose of BoTx led to the death of the rats on the third or fourth day after the injection. The neuromuscular block produced by the dose of BoTx used was probably the maximal block that can be produced by BoTx, since increasing the dose 20 times did not further increase the block as observed in dying rats that had received such a high dose. Most of the registrations were from superficial muscle fibres, but all fibres in the EDL muscle on the injected leg were paralysed as revealed by single fibre electromyography and recordings of mechanical muscle contraction. The method of injection used, did not produce obvious paralysis of the contra-lateral leg, but examination of the EDL muscle of this leg by single fibre EMG *in vivo* and the recording of epps *in vitro*, demonstrated that a large percentage of its muscle fibres were paralysed. The EDL muscle of the contra-lateral leg was used in studying a more mildly intoxicated muscle.

Conventional techniques with glass capillary microelectrodes were used for intracellular recording, a Medelec electromyography needle (SF 25) for the recording of single fibre action potentials during stimulation of the motor nerve, and a Grass transducer for the recording of isometric muscle tension. In the experiments with focal depolarization of the motor nerve terminal, a small intramuscular nerve branch was isolated and by microdissection traced to a point close to a group of superficial junctions. The nerve was then sucked into a glass capillary tube and current pulses applied through chlorided silver wires, one in the tube and the other one in the muscle bath.

To examine neuromuscular transmission in the patient with Eaton-Lambert syndrome, the compound muscle action potential was recorded by surface electrodes on the thenar and hypothenar muscles during single and repetitive stimulation of the corresponding motor nerve.

Further details of the experimental procedures are presented in the individual papers.

RESULTS

Effects of BoTx on transmitter release (Papers I, II)

BoTx does not completely block transmitter release from nerve terminals but mepps of reduced frequency and amplitude persist even when neurally evoked epps are abolished. It was found that mepps underwent gradual changes with time after poisoning. Initially frequency and amplitude of mepps were reduced, and amplitude histograms at individual endplates showed a skew, non-Gaussian, distribution with a predominance of small mepps. Both frequency and amplitidue of mepps increased with time after poisoning, and many mepps with normal or larger than normal amplitude appeared. Many of the mepps had prolonged rise times which implied that the exocytotic process of transmitter release might be disturbed. Experiments were performed to determine if the mepps recorded at BoTx poisoned endplates, as at normal endplates, represented spontaneous release of acetylcholine from nerve endings. It was shown that mepps did not appear if the muscle was denervated 1 day before poisoning, and that they were abolished by d-tubocurarine. This indicates that the mepps originated from the motor nerve and were caused by acetylcholine. It was also shown that the large mepps did not result from spontaneous regenerative activity in the nerve, as tetrodotoxin, which blocks action potentials, had no effect on the mepp amplitude distribution.

Contrary to previous reports, it was found that transmitter was released by nerve impulses at all endplates. But epp amplitude was greatly reduced and, at low stimulation frequency (0.5 Hz), 95% of the nerve

impulses at each endplate failed to release any transmitter when the ambient calcium concentration was 2 mM. When the extracellular calcium concentration was raised to 4 mM, the failure rate decreased to about 50% at most endplates. It was of interest that during the first 2 days after poisoning, epps at many endplates were of the same amplitude as the mepps of reduced size appearing after BoTx poisoning, and which also sometimes are seen at normal endplates (Cooke & Quastel 1973, Kriebel & Gross 1974). At later stages after poisoning, epp amplitude increased to the size of mepps in normal muscle, and the percentage of nerve impulses which failed to release transmitter decreased with time. But, during the whole observation period of three weeks, epps remained subthreshold and did not increase above the size of one or a few quanta. Unlike the mepps, the epps had normal time course at all endplates.

Mepps and epps were also examined in BoTx poisoned muscles that were only mildly intoxicated and not completely paralysed. In these muscles mepps in most fibres were of reduced amplitude and frequency similar to mepps in completely paralysed muscles, but epps showed all degrees of abnormality from many endplates with a large percentage of failures to release transmitter on nerve stimulation to some endplates with normal transmitter release. Single fibre electromyography in these muscles demonstrated an increase of the so-called neuromuscular jitter, suggesting a correlation between endplate potential amplitude and the jitter value.

It was possible, at all stages after poisoning, to enhance the frequency and normalize the time course and amplitude distribution of mepps to that at normal endplates. This could be done by procedures which enhanced spontaneous transmitter release, i.e. tetanic nerve stimulation, application of Black Widow Spider Venom or the calcium-ionophore A23187 or by mechanical irritation of the nerve terminal. These observations indicate, that in BoTx poisoning the nerve terminals contain normal transmitter quanta, but that the mecha-

nism for spontaneous transmitter release is disturbed.

To study if nerve evoked transmitter release after BoTx poisoning occurs in the form of preformed packets of acetylcholine as at normal endplates (del Castillo & Katz 1954), it was necessary to analyse if release followed Poisson statistics. The mepps, having a non-Gaussian amplitude distribution, did not consist of a uniform population of transmitter packets, and if evoked transmitter release followed Poisson statistics only part of the population of spontaneously released transmitter packets would be available for release by nerve impulses. It was possible to show, that if the mepps of normal amplitude distribution released during the action of Black Widow Spider Venom was used as quantum size, evoked transmitter release fitted Poisson's statistical law. It was also shown, that if statistical analysis was applied to the epp amplitude histogram at any stage after BoTx poisoning, there was a satisfactory agreement with the assumptions of Poisson's theorem. These results imply, that after BoTx poisoning, nerve evoked transmitter release is still quantal in nature and qualitatively similar to that at normal endplates.

Mode of action of BoTx (Paper I)

Since transmitter release is a calcium requiring process (see review by Rahamimoff 1976), it was of interest to examine the effect of calcium ions on the release process in BoTx poisoned nerve terminals. Raising the extracellular concentration of calcium, which in normal muscle increased the frequency of mepps, failed to affect the frequency or amplitude of mepps in BoTx poisoned muscles. This demonstrated an insensitivity to calcium, which could be caused either by a decreased calcium permeability of the nerve terminal membrane or by an intracellular insensitivity to calcium. To discriminate between these possibilities intracellular calcium was raised by different proce-

dures. Exposure of the nerve terminals to ouabain or to K-free solution, is thought to lead to an increase of intracellular calcium, and in normal muscle causes a marked increase of mepp frequency (Elmqvist & Feldman 1965, Birks & Cohen 1968). In BoTx poisoned muscles, however, these procedures did not influence transmitter release, which could indicate that nerve terminals in these muscles have a reduced sensitivity to intracellular calcium. This idea was supported by experiments with the calcium-ionophore A23187, which allows the passage of calcium ions across biological membranes. At normal endplates the application of the ionophore (10^{-5} M) led, after a delay of 10-20 minutes, to a massive release of transmitter. In BoTx poisoned muscles, the ionophore failed to cause this high frequency burst of mepps unless the extracellular calcium concentration was raised above 4 mM. Perfusion of the endplate region in BoTx poisoned muscles with 15 mM calcium, in the presence of the ionophore, caused a large increase of mepp frequency, and when the pipette was withdrawn the frequency returned to the initial low value.

These experiments demonstrated, that facilitating the entry of calcium ions across the nerve terminal membrane by the ionophore, did not by itself influence transmitter release in BoTx poisoning. If, however, at the same time, the extracellular calcium concentration was increased, allowing an augmented influx of calcium ions, spontaneous transmitter release similar to that which occurs in normal muscle at a lower level of calcium was obtained. These observations indicate that in BoTx poisoning the mechanism for spontaneous transmitter release has a reduced sensitivity to calcium, and if the intracellular concentration of calcium in the nerve terminal is raised, transmitter release can be normalized.

Nerve evoked transmitter release is probably triggered by the entry of calcium ions through the presynaptic membrane during the nerve terminal action potential (see review by Rahamimoff 1976). Evoked trans-

mitter release at BoTx poisoned endplates had a reduced sensitivity to the extracellular concentration of calcium, and the slope value for calcium dependence of transmitter release was about half that at normal endplates. By the addition of drugs that prolong the duration of the nerve action potential, it was thought possible to enhance the entry of calcium ions into the nerve and perhaps to antagonize the neuromuscular block in BoTx poisoning. This suggestion was tested by adding tetraethylammonium (TEA), a drug that blocks membrane potassium channels (Hille 1967). It was found that TEA, in the presence of 4 mM calcium, enhanced the epp amplitude *in vitro* and restored neuromuscular transmission producing normal twitch and tetanic tensions in previously completely paralysed muscles. TEA also normalized the calcium dependence of evoked transmitter release and normal or above normal slope values were obtained. These experiments show, that nerve evoked transmitter release, as in the case of spontaneous release, has a reduced sensitivity to calcium, and transmitter release can be restored, if the calcium concentration inside the nerve terminal is increased by enhancing calcium influx.

Restoration of transmitter release in BoTx poisoning
(Papers II, III)

The discovery that the block of neuromuscular transmission in BoTx poisoning is caused by a defect in the calcium mechanism of transmitter release, and that transmission was restored by TEA, led to attempts to find other antagonists of the paralysis. Guanidine, which increases evoked transmitter release from normal nerve terminals (Otsuka & Endo 1960, Kamenskaya, Elmqvist & Thesleff 1975), has been reported to be beneficial in human BoTx poisoning and to have some effect in animal intoxication (Cherington & Ryan,1968, 1970, Scaer, Tooker & Cherington 1969). It was found that guanidine, like TEA, restored neuromuscular transmission

in BoTx poisoning *in vitro* in the presence of 4 mM Ca^{2+}. To test if TEA and guanidine were equally effective *in vivo*, the techniques of single fibre electromyography (SFEMG) was used on paralysed animals. The drugs were found ineffective on completely paralysed muscles *in vivo*, but antagonized the block in muscles that were not completely paralysed, as evidenced by the decrease of neuromuscular jitter on SFEMG.

A possible explanation for the discrepancy between the restorative effects on neuromuscular transmission of TEA and guanidine *in vitro* and *in vivo*, could be, that the extracellular calcium concentration *in vitro* was kept at 4 mM, while *in vivo* it is around 2 mM. This hypothesis was tested by the recording of mechanical twitch tensions in response to nerve stimulation *in vitro* at different calcium concentrations. The restorative effect of TEA and guanidine on the paralysis in BoTx poisoned muscles was dependent on the ambient calcium concentration, and little restorative action was observed at 2 mM calcium.

At this stage another drug, 4-aminopyridine (4-AP), was tested. This drug, like TEA, is known to block potassium currents in excitable membranes (Pelhate & Pichon 1974, Yeh, Oxford, Wu & Narahashi 1976), and to antagonize the neuromuscular block produced by d-tubocurarine (Lemeignan & Lechat 1967). 4-AP, like TEA and guanidine, restored neuromuscular transmission *in vitro* in BoTx paralysed muscles, but unlike TEA and guanidine, the restorative effect was found to be independent of the extracellular calcium concentration in the range between 2 and 8 mM. By the *in vivo* recording of mechanical muscle tension in response to nerve stimulation, it was found that the intravenous injection of 4-AP, unlike TEA and guanidine, restored neuromuscular transmission from complete paralysis by BoTx to a normal level, recorded as normal twitch and tetanic tensions in the rat EDL muscle. 4-AP was also effective in restoring muscle tone and general locomotor activity in rats that were dying after a lethal dose of BoTx.

Effects of 4-aminopyridine on neuromuscular transmission (Paper IV)

Because of the powerful action of 4-AP as an antagonist to the neuromuscular block in BoTx poisoning, it was of interest to study its effects on normal neuromuscular junctions. 4-AP seemed to have no postsynaptic action as evidenced by its lack of effect on amplitude and time course of mepps in the rat EDL muscle or in the frog sartorius muscle. In rat muscle, micromolar concentrations of 4-AP increased transmitter release 5-10 times in response to single nerve impulses, as calculated from its potentiating effect on epp amplitude at d-tubocurarine blocked endplates. To estimate the increase of quantum content of epps, it was necessary to make a statistical analysis of the variance of epp amplitude (Elmqvist & Quastel 1965), as mepps could not be recorded in the presence of curare. Assuming that quantal release of transmitter is fluctuating according to Poisson's law, mean quantum content, m, was calculated from $m = \frac{\text{mean epp}}{q}$, where quantum size (q) was obtained from $q = \frac{\text{epp variance}}{\text{epp mean}}$, all values being corrected for non-linear summation (Martin 1955). In five endplates m was estimated before and after the application of 130 µM 4-AP, and was found to increase about 10 times from a mean quantum content of 196 $\pm$ 86 ($\pm$ S.D) to 2017 $\pm$ 507. However, transmitter release can deviate considerably from Poisson's statistics when quantum content is high (del Castillo & Katz 1954, Wernig 1975), and this method of calculation therefore represents only an approximate estimate of quantal release.

4-AP also enhanced transmitter release during repetitive nerve activity but, at d-tubocurarine blocked endplates, only the first impulses caused increased transmitter release at stimulation frequencies at or above 50 Hz. At magnesium blocked endplates, 4-AP potentiated transmitter release at every stimulus during tetanic nerve stimulation and, as in BoTx poisoning, restored neuromuscular transmission.

TABLE I

Influence of 4-AP on the statistical parameters of transmitter release. Transmission is blocked by magnesium and m, n and p were determined at individual endplates before (control) and after the addition of 4-AP. At each endplate 150 to 300 mepps and epps (0.1 Hz) were recorded, and the statistical parameters were determined by the formulas given in the text. Endplates with initial values of p less than 0.20 were rejected because of the large error associated with the estimate at such endplates (Bennett, Florin & Hall 1975).

m	n	p	conc.	conc.
control/4-AP	control/4-AP	control/4-AP	4-AP (μM)	Mg^{2+} (mM)
13.8/21.1	66/162	0.21/0.13	1	10
59/170	236/810	0.25/0.21	3	10
94/223	427/769	0.22/0.29	2.5	15
13/25	53/117	0.25/0.21	2	5

Transmitter release from motor nerve terminals has been shown to obey binomial statistics (Johnson & Wernig 1971, Bennett & Florin 1974), and if the average number of quanta released per nerve impulse (m) is given by $m = np$, p can be calculated from $p = 1 - \frac{\text{epp variance}}{\text{epp mean}} \cdot \gamma + \frac{\delta^2}{\gamma^2}$ (Bennett & Florin 1974), where γ is the mean amplitude of the mepps and δ the variance of mepps, all values corrected for nonlinearity of the endplate response (Martin 1955). The statistical parameters p and n are thought to represent physiological events in the release process. p may represent the probability of release of available transmitter quanta, and n the store of transmitter available for release (Liley & North 1953, Elmqvist & Quastel 1965, Bennett & Florin 1974) or the number of active release sites in the presynaptic region (Zucker 1973, Wernig 1975). Statistical estimates of binomial parameters of transmitter release have recently been subject to criticism, as there may be spatial or temporal variation in the probability of release creating errors in these estimates (Brown, Perkel & Feldman 1976, Barton & Cohen 1977). However, the effects of 4-AP on these parameters were studied in frog muscle, and it was found that 4-AP increased m by increasing n and not p (Table I), as has previously been shown for TEA and guanidine (Volle & Branisteanu 1976). It should be noted that the maximal values obtained for n, between 500 and 1000, correspond with the number of active release sites estimated for the frog neuromuscular junction (Heuser 1976).

Mode of action of 4-aminopyridine (Papers III, IV)

As mentioned above 4-AP, like TEA, blocks potassium currents in excitable membranes (Yeh, Oxford, Wu & Narahashi 1976), and it could therefore be expected to prolong the nerve terminal action potential, and in that way enhance the entry of calcium into the terminal during the nerve impulse. Since it was impossible to

accurately measure the time course of the nerve terminal action potential, the effects of 4-AP were studied on the muscle action potential. In concentrations that maximally antagonized the paralysis of BoTx, 4-AP doubled the duration of the muscle action potential. In this respect 4-AP and TEA were equally active, while guanidine caused only a slight prolongation of the action potential.

The 5-10 times increase of transmitter release by 4-AP at single nerve impulses in d-tubocurarine blocked muscles, represents a very great potentiation, compared with the increase of about 1.5 to maximally 3 times produced by previously studied drugs like TEA and guanidine (Koketsu 1958, Kamenskaya, Elmqvist & Thesleff 1975). This enormous potentiation of transmitter release prompted further investigations on the mode of action of 4-AP. To study the depolarization-secretion coupling in the motor nerve terminal, conducted nerve impulses were blocked by tetrodotoxin or by the removal of extracellular sodium. Under these conditions, electrotonic depolarization of the nerve terminal in the absence of drugs, causes stimulus graded release of transmitter (Katz & Miledi 1967). The addition of 4-AP converted the graded epp to an all-or-none type of response with an epp of 50-60 mV amplitude being triggered over a narrow range of depolarizing current. This type of effect has previously been observed for TEA (Katz & Miledi 1969), and it was of interest that guanidine also elicited a similar type of response, suggesting a common mode of action for all three drugs. It was concluded that the large epps triggered by 4-AP, TEA and guanidine were induced by a regenerative calcium influx into the nerve terminal, since these epps appeared only in the presence of calcium and were completely abolished by manganese, which blocks membrane calcium currents.

The molecular events by which 4-AP affects calcium influx in motor nerve terminals are unknown, but the drug was still effective when the temperature was decreased from 37^{o} to 15^{o}C, suggesting that its action is independent of metabolic energy.

The time course of drug action was investigated by ionophoretic microapplication of 4-AP to single nerve terminals. The delayed onset and prolonged duration of action demonstrated by this method, explains the observation, that after addition of the drug to the bathing solution, it takes a few minutes before the effect starts, and that after removal of the drug its effects are reversed very slowly over several hours.

4-AP produces, in the spinal cord of the cat (Lemeignan 1973) and in the abdominal ganglion of the cockroach (Hue, Pelhate, Callec & Chanelet 1976), repetitive spontaneous nerve activity. As spontaneous action potentials in the nerve can facilitate transmitter release, it was of importance to determine if this also occured at the neuromuscular junction. At frog endplates, but not in the rat, the addition of 4-AP caused repetitive epps in response to single nerve impulses. If the external calcium concentration was decreased below 0.1 mM, 4-AP in both frog and rat muscle promptly produced vigorous muscle twitches, which were abolished by d-tubocurarine and by tetrodotoxin. In denervated muscles this did not occur, which demonstrates that the muscle contractions were caused by spontaneous nerve activity.

4-aminopyridine tested in the treatment of Eaton-Lambert syndrome (Paper V)

4-AP is a potent drug to increase transmitter release from motor nerves, and as it might be of value in the treatment of neuromuscular diseases, it was tested in man. It has previously been used in anaesthesiology as an anticurare agent (Paskov, Staenov & Mirov 1973), and it was reported that it could be given intravenously as single-dose injections to humans without side effects.

It was of interest to test 4-AP in the myasthenic syndrome sometimes associated with bronchogenic carcinoma (Eaton-Lambert) as muscular weakness in that di-

sease, as in BoTx poisoning, is caused by diminished release of acetylcholine from motor nerve terminals (Elmqvist & Lambert 1968). It was considered important to try new drugs in the treatment of this disease, because the conventional treatment by guanidine (Lambert 1966) can cause serious side effects (Henriksson, Nilsson, Rosén & Schiller 1977).

The intravenous injection of 4-AP chloride to a patient suffering from the Eaton-Lambert syndrome restored neuromuscular transmission in a dose-dependent manner. The drug was also active by oral administration, and the optimal dose and approximate duration of action was determined by performing electrophysiological examinations after repeated oral doses of 4-AP hydrochloride. The patient was given 4-AP continuously by the oral route for fourteen days, and during this time his muscle strength recovered and neuromuscular transmission was restored to almost normal level. During treatment with 4-AP the patient reported increased wakefulness, and with a dose of 4-AP sulphate of more than 100 mg/day the patient complained of photophobia and a slight sensation of unsteadiness during walking. However, neuro-ophtalmological examination and recording of the visually evoked response were normal, and clinical examination did not demonstrate any objective disturbance of co-ordination, posture or gait. The subjective symptoms experienced by the patient disappeared when the dose was reduced. Since it is not known, if 4-AP has long-term toxic effects the treatment was terminated after a few weeks. Upon withdrawal of the drug the patient's muscle weakness reappeared.

DISCUSSION

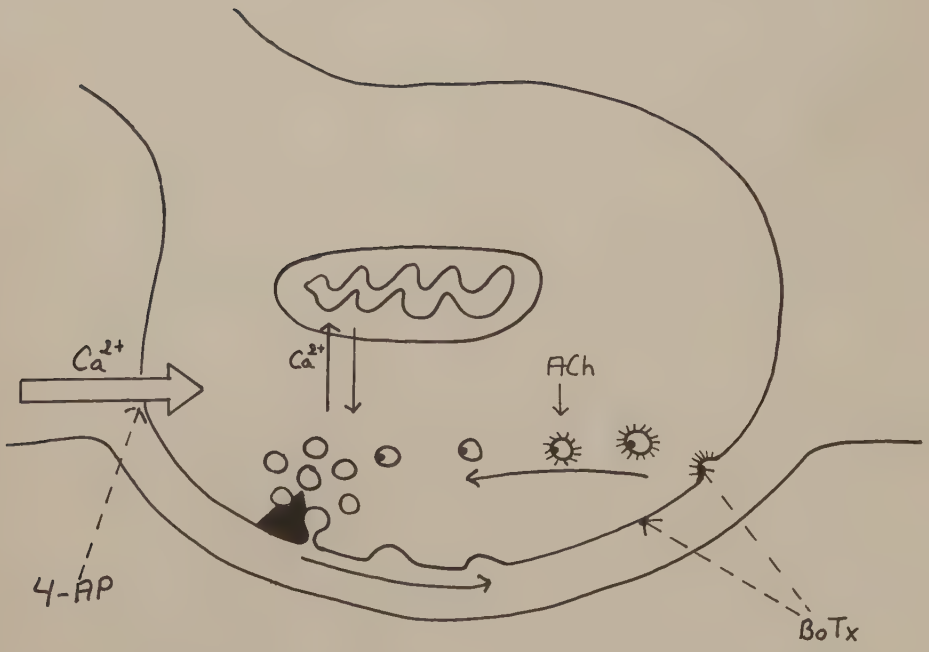
Neuromuscular transmission and the mode of action of BoTx

During the 1970s significant advances have been made in the understanding of the mechanisms involved in neuromuscular transmission both at the pre- and postsynaptic level. Postsynaptically, the analysis of endplate noise (Katz & Miledi 1972) made it possible to describe the effects of acetylcholine and drugs on individual ionic channels involved in the transmitter action on the postsynaptic membrane. By the observation that certain snake neurotoxins bind irreversibly to the acetylcholine receptor (Chang & Lee 1963, 1966), it became possible to morphologically localize the receptor on the muscle membrane and also to isolate and purify the receptor molecule (see review by Fulpius 1976). It also became possible to produce an animal model of myasthenia gravis (experimental autoimmune myasthenia; see review by Heilbronn 1976) and, clinically, major advances were made concerning the etiology of myasthenia gravis, and new ideas of how to treat that disease were presented (Engel & Warmolts 1971, Mann, Johns, Campa & Muller 1976).

Presynaptically there has been a similar expansion of knowledge concerning the transmitter release mechanism. The nerve terminal is filled with membrane-bound vesicles (Palade 1954) which are believed to represent the quantal packets of transmitter (del Castillo & Katz 1955), and in recent years the physiological steps involved in the transmitter release process have been correlated with morphological structures at the nerve terminal (see review by Heuser 1976).

At the nerve terminal membrane, synaptic vesicles are concentrated and arranged in parallel rows at specialized membrane release sites localized at regular intervals opposite to the transverse folds of the postsynaptic membrane (see fig.). It is believed that the exocytotic process of transmitter release is initiated by influx of calcium ions into the nerve terminal (see review by Rahamimoff 1976), and that this entry of calcium is caused by a voltage dependent increase of calcium permeability of the nerve terminal membrane which occurs during depolarization by the nerve action potential. The molecular reactions involved in this process or in the process of vesicle discharge of transmitter are yet unknown, but it has been suggested that calcium may neutralize the electronegativity on the surface of the vesicles and nerve terminal membrane, and by that eliminate the electrostatic repulsion between them (Blioch, Glagoleva, Liberman & Nenasheo 1968). By the use of electron microscopy and particularly the technique of freeze-fracturing, it has been shown that vesicle discharge involves the fusion of vesicles with the preterminal membrane, and the vesicle membrane becomes part of the terminal membrane. At the same time the terminal membrane is invaginated at other points, and the invaginates are pinched off to form new vesicles (see fig.). This membrane recycling is thought to represent the structural basis for quantal release of transmitter, and strong evidence points to the vesicle as the structural correlate of one quantum.

In the sequence of events in the transmitter release process at the motor nerve terminal, BoTx apparently interferes with the step that involves calcium. This was shown by a reduced sensitivity of the release process to alterations of the extracellular calcium concentration, and its restoration by procedures that increase the level of intracellular calcium, i.e. tetanic nerve stimulation, application of the calcium-ionophore and drugs that increase the influx of calcium. The results indicate a defect of the release process some-



Schematic summary of the current view of the mechanism of transmitter release at the neuromuscular junction and of the possible sites of action of BoTx and 4-AP. Transmitter release is triggered by the entry of calcium ions into the nerve terminal during the depolarization caused by the nerve action potential. Calcium neutralizes the electronegativity on the surface of the synaptic vesicle and the nerve terminal membrane, and thereby allows the vesicle to fuse with specialized "active zones" (dark triangle) in the plasma membrane. The acetylcholine content of the synaptic vesicle is released into the synaptic cleft during vesicle fusion. The vesicle membrane is incorporated into the nerve terminal membrane, and vesicles are reformed by subsequent endocytic invagination and budding off of new vesicles from the nerve axolemma. The reformed vesicles are recharged with newly synthesized acetylcholine (ACh) in the nerve terminal.

It is suggested that BoTx binds to the nerve terminal membrane and enters the terminal by the endocytosis accompanying vesicle recycling. The intracellular uptake of BoTx blocks, in an unknown way, the mechanism of transmitter release by decreasing its sensitivty to calcium.

4-AP is suggested to act on a voltage dependent calcium permeability in the nerve terminal membrane, and thereby increase calcium influx during the nerve terminal action potential. The enhanced concentration of free calcium inside the nerve ending induced by 4-AP, is sufficient to overcome the calcium insensitivity of the transmitter release mechanism caused by BoTx. It is believed that the excess of free calcium inside the nerve ending immediately after the nerve spike, is removed by being taken up by intracellular calcium binding organelles like mitochondria.

where between the influx of calcium ions and the discharge of the vesicle content of transmitter into the synaptic cleft. The mechanism by which BoTx reduces the intracellular sensitivity to calcium is unknown, as is the physiology of this step in the normal release process. It can however be concluded, that BoTx does not interfere with the formation of synaptic vesicles, as evidenced by the normal structural appearance of the vesicles after BoTx poisoning (Thesleff 1960). However, it is reasonable to speculate, that the action of BoTx might be dependent on the endocytotic process involved in membrane recycling at the nerve terminal. As pointed out the onset of action of BoTx is delayed after binding of the toxin to the nerve endings, and the latency period is dependent on nerve activity and metabolic energy (see introduction). All these observations might be explained if BoTx has an intracellular site of action, entering the nerve terminal by endocytosis and, perhaps, being incorporated into the membranes of the synaptic vesicles (see fig.).

The observations that in BoTx poisoning quantal release of transmitter is disturbed for spontaneous release but qualitatively functional during nerve evoked release, cannot be explained by the data obtained in this investigation. It was found, that spontaneously released transmitter packets were not uniform in size, and only some of them also took part in nerve evoked release. It is possible that the mepps of reduced amplitude represent gated release of transmitter, i.e. fractional depletion of the vesicles involved in the transmission process (see Pfenninger 1973). It may be relevant that small mepps have been described at normal endplates (Cooke & Quastel 1973, Kriebel & Gross 1974). Small mepps in normal muscles occur with a frequency similar to the small mepps seen in early stages of BoTx poisoning, and they may represent release of transmitter insensitive to calcium. These small packets of acetylcholine were released by nerve stimulation during the first and second day after BoTx poisoning, but at later stages only normal sized quanta of transmitter

were released by nerve impulses. The increase of mean mepp amplitude and the reduction of the number of failures of epps with time after poisoning, may to some extent be caused by nerve activity. This is suggested by the findings that tetanic nerve stimulation for several hours during early stages of BoTx poisoning produced similar alterations of mepps and epps. Repetitive nerve stimulation causes, by accumulation, increased concentration of intracellular calcium (see review by Rahamimoff 1976), but at normal nerve terminals, it is thought that this excess of free calcium is taken up by intracellular calcium binding organelles like mitochondria within seconds or minutes (see fig.). It is not known, if this calcium regulating function of intracellular organelles also includes a feed-back compensation for decreased transmitter release, but it is possible, that an increase of the intracellular concentration of calcium may provide a way for the nerve terminal to compensate for the effects of BoTx.

The giant mepps recorded at later stages after BoTx poisoning may be composed of several quanta of transmitter. This would explain their prolonged time course and also the steps sometimes observed on their rising phases. As mentioned above, the ultrastructural picture of BoTx poisoned endplates shows normal synaptic vesicles, which seems to exclude the possibility that the large mepps were caused by variability of vesicle size as a result of a disturbance of the recycling mechanism of vesicles which has been demonstrated to occur in other conditions (Cull-Candy, Fohlman, Gustavsson, Lüllmann-Rauch & Thesleff 1976, see also review by Heuser 1976).

Restoration of BoTx paralysis by drugs

The paralysis produced by BoTx was antagonized by tetraethylammonium (TEA), guanidine and 4-aminopyridine (4-AP). The restorative effect on neuromuscular transmission of TEA and guanidine was dependent on the level

of extracellular calcium, and the finding that these two drugs had hardly any restorative effect at 2 mM Ca^{2+}, may explain their inability to restore neuromuscular transmission *in vivo* in fully paralysed muscles. TEA and guanidine, however, improved neuromuscular transmission *in vivo* in muscles that were not completely paralysed, as shown by their effect on the neuromuscular jitter in single fibre electromyography. The effect of 4-AP, on the other hand, was independent on the extracellular level of calcium, and this drug was equally effective in restoring neuromuscular transmission *in vitro* and *in vivo*. The reason for the difference in calcium dependence between the drugs is not known.

TEA and 4-AP were initially tested because they were expected, by their blocking effect on membrane potassium currents (Hille 1967, Yeh et al. 1976), to prolong the nerve terminal action potential, and in that way increase the influx of calcium into the nerve. It was not possible to measure directly the time course of the nerve terminal action potential, and therefore the effects of the drugs were studied on the muscle action potential. TEA and 4-AP markedly prolonged its time course, but guanidine caused only a slight prolongation. These results were thought to indicate that the mode of action of guanidine is different from that of TEA and 4-AP. Since guanidine is known to inhibit the binding and uptake of divalent cations to subcellular organelles,it was suggested that guanidine enhanced transmitter release by allowing free calcium to accumulate inside the terminal by interfering with intracellular binding of calcium to organelles such as the mitochondria.

As it was not possible, from these results, to draw any firm conclusions regarding the mode of action of 4-AP, TEA and guanidine, new experimental methods were tried. Transmitter release was studied in the absence of conducted nerve impulses, and this experimental method made it possible to release transmitter by graded depolarization of the nerve terminal (Katz & Miledi

1967). It was found that all three drugs converted the stimulus-graded transmitter release that occured in the absence of drugs, to an all-or-none type of release, producing an enlarged endplate potential over a narrow range of stimulus currents. This drug effect was dependent on the presence of extracellular calcium, and occured even in the absence of all ions except calcium, and it was abolished by manganese, which blocks membrane permeability to calcium. It was therefore concluded that all three drugs caused a regenerative calcium influx into the nerve terminal. These effects of the drugs on calcium permeability of the nerve makes it reasonable to suggest, that the drugs also under normal conditions increase transmitter release by increasing the influx of calcium. Such a mode of drug action represents a new type of pharmacological mechanism at the nerve terminal, and it might be of general importance, since it offers a possibility to study the role of calcium in the transmitter release mechanism.

The mechanism by which 4-AP, TEA and guanidine induce the regenerative calcium current is not known. Such an effect may result from blockade of potassium efflux, which could be sufficient to enable a small inward current of calcium to become regenerative. As mentioned above, 4-AP and TEA are known to block membrane potassium channels. However, guanidine which did not markedly prolong the muscle action potential, and which has no known effect on outward potassium current (Narahashi, personal communication), also produced the same kind of regenerative calcium dependent response. It is therefore possible, that all three drugs act directly on voltage-sensitive calcium channels in the nerve terminal membrane allowing the passage of larger calcium currents. The presence of a voltage dependent calcium permeability in the nerve terminal membrane could, as suggested by Jankowska, Lundberg, Rudomin & Sykova (1977), be part of a basic physiological mechanism modulating transmitter release at synapses in the peripheral and central nervous system.

Testing 4-aminopyridine in man

4-AP had already been tried in man as an anticurare agent in anaesthesiology (Paskov, Staenov & Mirov 1973), and it was of interest to test it in human neuromuscular diseases. Since 4-AP acts by increasing transmitter release, it could be expected to be particularly effective in diseases where muscle weakness is caused by reduced transmitter release, as in the myasthenic syndrome sometimes associated with bronchogenic carcinoma (Eaton-Lambert).

It is not known if the reduction of nerve evoked transmitter release in the Eaton-Lambert syndrome, as in BoTx poisoning, is caused by a decreased sensitivity to intracellular calcium in the nerve terminal. There are differences in the pathophysiology of the Eaton-Lambert syndrome and BoTx poisoning. Microelectrode recordings from intercostal muscle endplates, obtained from patients with the Eaton-Lambert syndrome, have shown that mepps in this disease have normal amplitude and frequency (Elmqvist & Lambert 1968, Lambert & Elmqvist 1971), which indicates a true difference between the two conditions. In human BoTx poisoning mepps of reduced amplitude and frequency have been described (Lambert, Engel & Cherington 1974), similar to the findings in experimental BoTx poisoning in the rat.

It was shown that 4-AP restored neuromuscular transmission in the Eaton-Lambert syndrome, and that the drug was effective when given continuously orally for three weeks. No serious side effects were observed. 4-AP can also improve neuromuscular transmission in myasthenia gravis (unpublished) where muscular weakness is believed to be caused by mainly a postsynaptic disorder. It is possible that 4-AP, in the future, may be a valuable drug in the treatment of several human neuromuscular diseases, and perhaps also in other conditions with disturbed synaptic transmission in the peripheral and central nervous system.

SUMMARY

The main results of the present studies are:

that botulinum toxin (BoTx) does not completely block spontaneous transmitter release from motor nerve terminals. Miniature endplate potentials (mepps) are recorded at all endplates, their frequency is reduced and their amplitude distribution abnormal. Raising the extracellular calcium concentration increases the frequency of mepps in normal muscle but not in BoTx poisoned ones. Procedures which are believed to raise the intracellular calcium level in the nerve terminals, i.e. exposure to potassium free solution or to ouabain, similarly increase mepp frequency in normal but not in poisoned muscles. When the calcium-ionophore A23187, which allows the passage of calcium ions through biological membranes, is applied together with high external calcium (> 4 mM), the frequency of mepps is enhanced and their amplitude distribution altered towards a normal Gaussian one with a mean mepp size approaching that at normal endplates.

that BoTx does not completely block neurally evoked transmitter release. Endplate potentials (epps) are recorded at all endplates. Mathematical analysis indicates that transmitter release occurs in the form of preformed quanta of acetylcholine as at normal endplates, quantum size being initially reduced but after a few days approaching normal size. Epps are made up of single or a few transmitter quanta and have a high failure rate. The extracellular calcium concentration which causes a certain level of transmitter release in response to nerve impulses is considerably higher at

BoTx poisoned endplates than at normal ones. The slope value for calcium dependence of transmitter release is about half of that at normal endplates.

that tetraethylammonium and guanidine, in concentrations of about 3 mM, restore neuromuscular transmission *in vitro* from complete paralysis by BoTx to about normal level, provided that the external calcium concentration is 4 mM or higher. The two drugs are ineffective on completely paralysed muscles *in vivo*, but improve neuromuscular transmission in muscles that are not completely paralysed as demonstrated by single fibre EMG. 4-Aminopyridine also restores neuromuscular transmission following paralysis by BoTx but is about 20-30 times more potent than tetraethylammonium and guanidine. Unlike these drugs, 4-aminopyridine is effective when the ambient calcium concentration is 2 mM and therefore also active *in vivo*. In rats paralysed by a lethal dose of BoTx, the intraperitoneal administration of 4-aminopyridine restores general motor activity, the effect lasting 1-2 hours.

that tetraethylammonium, guanidine and 4-aminopyridine increase nerve evoked transmitter release by enhancing calcium influx during depolarization of the nerve terminal, probably by affecting a voltage dependent calcium permeability in the nerve membrane. This represents a novel type of pharmacological mechanism.

that 4-aminopyridine restored neuromuscular transmission to almost normal level in a patient with the myasthenic syndrome associated with bronchogenic carcinoma (Eaton-Lambert), and that the drug was effective when given orally for three weeks.

It is suggested that in BoTx poisoning the transmitter release mechanism has a reduced sensitivity to calcium and that its activation requires an elevated level of intracellular calcium. When the intracellular concentration of calcium in the nerve terminals is raised

to a higher than normal level, by the application of the calcium-ionophore in combination with high external calcium or by drugs which enhance calcium influx during the nerve impulse, the transmitter release mechanism is activated and approximately normal amounts of transmitter are released.

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